

Cochlin Produced by Follicular Dendritic Cells Promotes Antibacterial Innate Immunity

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SUMMARY

Cochlin, an extracellular matrix protein, shares homologies with the Factor C, a serine protease found in horseshoe crabs, which is critical for antibacterial responses. Mutations in the *COCH* gene are responsible for human DFNA9 syndrome, a disorder characterized by neurodegeneration of the inner ear that leads to hearing loss and vestibular impairments. The physiological function of cochlin, however, is unknown. Here, we report that cochlin is specifically expressed by follicular dendritic cells and selectively localized in the fine extracellular network of conduits in the spleen and lymph nodes. During inflammation, cochlin was cleaved by aggrecanases and secreted into blood circulation. In models of lung infection with *Pseudomonas aeruginosa* and *Staphylococcus aureus*, *Coch*^{-/-} mice show reduced survival linked to defects in local cytokine production, recruitment of immune effector cells, and bacterial clearance. By producing cochlin, FDCs thus contribute to the innate immune response in defense against bacteria.

INTRODUCTION

Cochlin, encoded by the gene *Coch*, is a highly abundant extracellular matrix protein in the cochlea and vestibule of the inner ear (Robertson et al., 1994; Robertson et al., 1997). Missense mutations and in-frame deletions in the *COCH* gene are etiologically linked to the autosomal-dominant DFNA9 syndrome, a disorder characterized by late onset nonsyndromic hearing loss and vestibular dysfunctions with associated neurosensory degeneration in the inner ear (Manolis et al., 1996). Whereas the neurodegeneration associated with DFNA9 is believed to be caused by a gain-of-function by the mutated cochlin, the normal physiological function of cochlin remains unknown. The N terminus of co-

chlin contains a LCCL domain also present in Factor C, a serine protease of the horseshoe crab *Limulus* involved in activating the coagulation cascade in response to LPS (Iwanaga et al., 1992). The expression of cochlin is highly selective; it is abundantly expressed in the inner ear and also found in the spleen (Robertson et al., 1997). To understand the physiological function of cochlin in the spleen, we investigated the splenic cell type that produces cochlin, the regulation of its production, and its possible role in immunity.

Follicular dendritic cells (FDCs), a type of stromal cells of the secondary lymphoid organs, are recognized as key organizers of B cell follicles and central to the development of germinal centers (GCs) where cooperation of multiple cell lineages leads to the formation of isotype-switched, high-affinity immunoglobulin and the establishment of humoral immune memory (Allen and Cyster, 2008). FDCs function by presenting native antigen in the form of immune complexes to B cells, supporting both T-dependent and -independent B cell activation, and suppressing apoptosis in B cells with high affinity antigen-binding receptors resulting from successful somatic hypermutation processes. In the secondary lymphoid organs, FDCs are tightly associated with a fine tubular extracellular matrix network—named conduits—used as an adhesion substrate for cell's crawling movement and involved in distributing small molecules through the splenic white pulp and lymph nodes, respectively (Bajénoff et al., 2006; Gretz et al., 2000; Nolte et al., 2003; Sixt et al., 2005). FDCs may on one hand secrete cytokines such as CXCL13 into the conduits and, on the other hand, capture small antigens circulating in the conduits (Roozendaal et al., 2009). Although the function of FDCs in modulating the activation of B cells can be regulated by innate immunity, FDCs have no established role in modulating innate immunity (El Shikh et al., 2007; Garin et al., 2010; Suzuki et al., 2010).

The homology of cochlin to the *Limulus* Factor C protein led us to postulate that cochlin may participate in host resistance to infection. In the present study, we investigated the role for cochlin in immunity. We demonstrate that cochlin is produced specifically by the FDCs in the secondary lymphoid organs and exclusively present in the fine extracellular network of conduits.

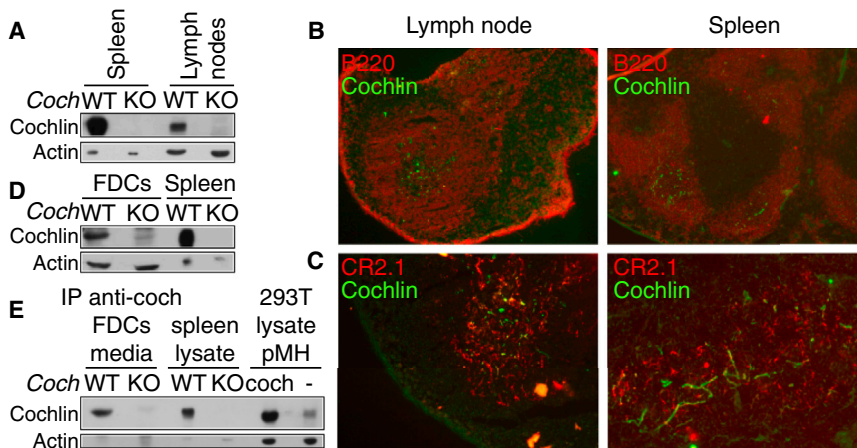


Figure 1. Cochlin Is Produced and Secreted by FDCs in the Spleen and the Lymph Nodes

(A) Spleen and lymph node lysates from WT and *Coch*^{-/-} mice were analyzed by WB.

(B and C) Lymph node and spleen sections from WT mice were costained for cochlin and B cell (B220) (objective lens 10X) (B) or FDCs (CR2.1) (objective lens 20X) (C).

(D and E) Primary FDCs were isolated from WT and *Coch*^{-/-} mice. Lysates from in vitro cultured FDCs were analyzed by WB (D). Culture media were subjected to anti-cochlin IP and analyzed by WB (E). Spleen lysates from WT and *Coch*^{-/-} mice, as well as lysates from 293T cells expressing cochlin were used as controls.

See also Figure S1.

However, cochlin deficiency has no effect on acquired immunity. Instead, we show that cochlin LCCL domain is released into the blood after proteolytic cleavage in response to LPS and bacterial infection. Finally, we provide evidence that cochlin deficiency leads to a defective innate response and an increased sensitivity to *P. aeruginosa* infection. Our study demonstrates cochlin as the first immune modulator released from conduits to act at the systemic level, revealing a unique biological function of conduits and evidence for a role of FDCs in regulating innate immunity.

RESULTS

Cochlin Is Expressed and Secreted by Follicular Dendritic Cells

We investigated the expression of cochlin by using a highly specific monoclonal antibody (Yao et al., 2010) and found that cochlin is expressed in spleens and lymph nodes (Figure 1A; see also Figure S1A available online). However, because both protein and messenger RNA (mRNA) of cochlin are not detectable in isolated splenocytes, which contain mostly B and T cells (Figure S1B), we considered the possibility that cochlin is expressed by stromal cells in the spleens. By using in situ immunocytochemistry, we found that cochlin is present in the fine network-like structure located in the B cell zone in the white pulp of spleens and in the center of the B cell follicles in lymph nodes (Figure 1B; Figure S1C). Because this localization corresponds to that of the follicular dendritic cells (FDCs), we examined the coimmunostaining of CR2.1, a marker for FDC, and cochlin. Indeed, cochlin is localized in the close proximity of the FDCs (Figures 1C). Cultured primary WT, but not *Coch*^{-/-}, FDCs express and secrete cochlin in vitro (Figures 1D and 1E). We thus conclude that FDCs express and secrete cochlin in the spleen and the lymph nodes.

Cochlin Accumulates in the Lumen of the Conduits

Because cochlin is an extracellular protein and distributed in a fine network-like structure in the spleen and lymph nodes (Figure 1), we considered the possibility that cochlin might be associated with the conduits, an extracellular matrix network involved in distributing chemokines and small blood and lymph-borne

molecules through the splenic white pulp and lymph nodes, respectively (Gretz et al., 2000; Nolte et al., 2003; Roozendaal et al., 2009; Sixt et al., 2005). We investigated whether cochlin colocalizes with known markers for conduits such as collagen, ER-TR7, and perlecan in the spleens. We found that cochlin colocalizes with the subset of conduits present in the B cell zone, but not in the periarteriolar lymphocyte sheath (PALS) (Figure 2A). Although most of the conduit constituents are synthesized by enveloping fibroblastic reticular cells (FRC), only conduits in the B cell zone are associated with FDCs in addition to FRCs (Gretz et al., 2000; Lokmic et al., 2008; Roozendaal et al., 2009; Sixt et al., 2005). The specific association of cochlin with the conduits in the B cell zone further supports that cochlin is produced by the FDCs.

Conduits are formed by a central collagen bundle core surrounded by a microfibrillar layer, which is wrapped in a basement membrane. High-resolution fluorescence microscopic analysis of splenic samples coimmunostained with anticochlin and antibodies for laminin-1 and perlecan, markers for the basement membrane, or with antibody for ER-TR7, a marker for the microfibrillar layer, demonstrate that cochlin is wrapped by the basement membrane and by the microfibrillar layer. Indeed, fluorescence microscopy analysis suggests that cochlin colocalizes with the central collagen core (Figure 2B). By using electron microscopic analysis, we found that cochlin colocalizes with the central collagen bundles and with fibrillar collagen in the conduits (Figure 2C). We thus concluded that cochlin is synthesized by the FDCs and secreted in the lumen of the FDCs-associated conduits.

We next assessed the function of cochlin by analyzing the FDC-associated conduits in the spleen and lymph nodes of *Coch*^{-/-} mice. Cochlin deficiency did not modify the FDC-associated conduit's structure as seen in fluorescent and electronic microscopy (Figures S2A–S2C). In the spleen or the lymph nodes, immune cells crawl on the surface of the conduits to circulate (Bajénoff et al., 2006), and cochlin deficiency did not impair B cell, FDCs or T cell localization in the spleen or the lymph nodes of *Coch*^{-/-} mice (Figures S2A and S2B; data not shown). Conduits also allow for the flow of low molecular weight molecules (MW ≤ 70 kDa) such as cytokines and small antigens (Gretz et al., 2000; Nolte et al., 2003; Roozendaal et al., 2009) in their lumen. Cochlin deficiency did not modify either the flow of small

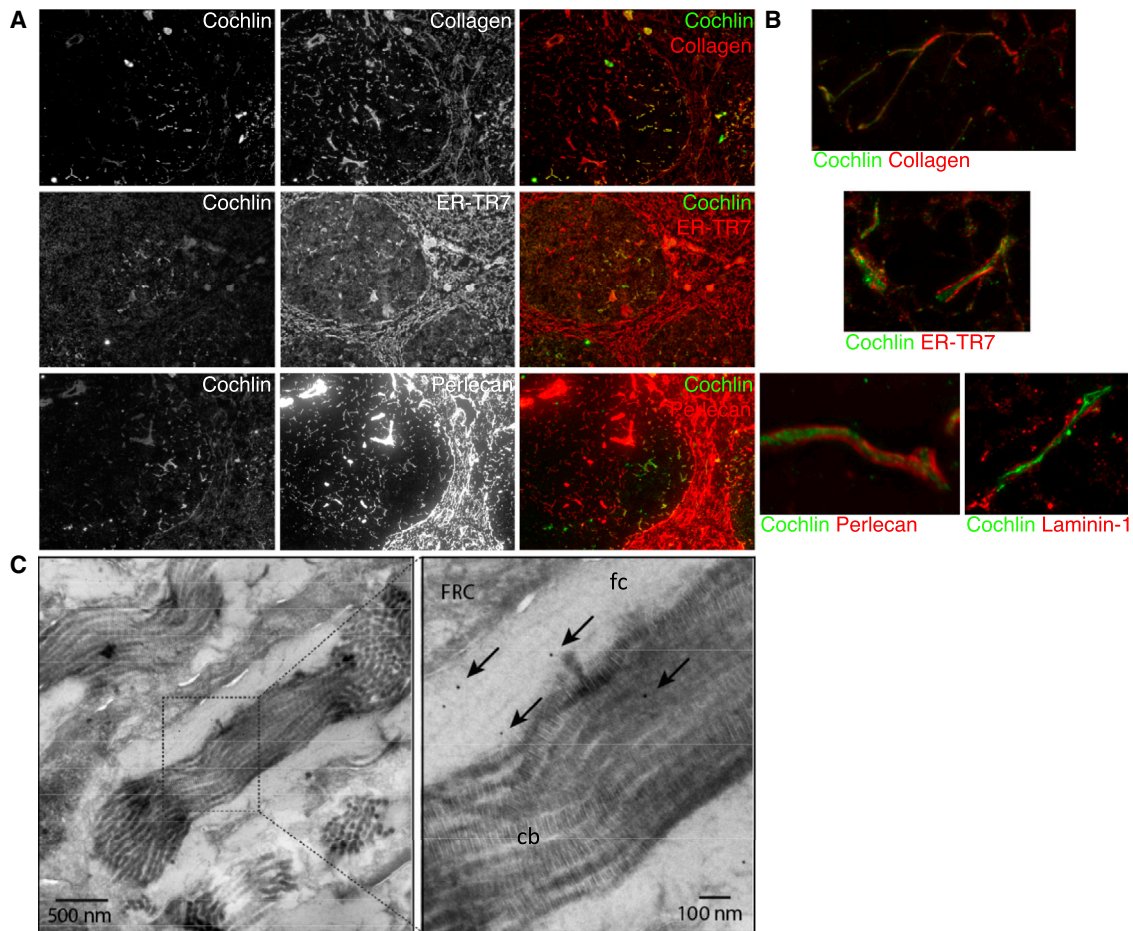


Figure 2. Cochlin Concentrates in the Lumen of Conduits

(A and B) Spleen sections from WT mice were costained for cochlin and the conduits markers collagen, ER-TR7, perlecan, and laminin-1. Objective lens 10X (A). Objective lens 100X, 3D reconstructions were performed by using deconvolved z series (B).

(C) Spleen fine sections were stained for cochlin (gold beads, black arrowheads; fc, fibrillar collagen; cb, collagen bundles).

See also Figure S2.

molecules or the molecular weight cutoff of the components circulating through the conduits, as observed by following the flow of 10, 70, and 500 kDa fluorescent dextran in *Coch*^{-/-} spleens (Figures S2D–S2F). FDCs adhere to the surface of the conduits and capture circulating small antigens before transferring them to the B cells, or alternatively B cells directly sample small antigens circulating in the conduits (Rozen daal et al., 2009). However, cochlin deficiency in vivo did not impair the close association of FDCs with the conduits (Figure S3A). Thus, we conclude that cochlin deficiency has no effect on the adherence of FDCs to the conduits.

We next tested whether cochlin was involved in the transfer of small antigens from the conduits to the immune cells, but cochlin deficiency did not alter the ability of FDCs or B cells to capture small antigens at their surfaces in the lymph nodes (Figures S3B and S3C). In addition, *Coch*^{-/-} mice did not show any defects in the immunoglobulin response to the small antigens TEL (turkey egg lysozyme, 12–14 kDa) trafficking through the conduits lumen nor to the large antigen PE (phycoerytherin, 240 kDa) excluded from the conduits, as anticipated, and used

here as a control (Figure S3D) (Rozen daal et al., 2009). Furthermore, *Coch*^{-/-} mice showed no defect in germinal center formation (Figure S3E) or antibody affinity maturation during primary and secondary responses (Figures S3F and S3G). Taken together, we conclude that although cochlin is specifically associated with the conduits in the B cell zone, cochlin deficiency has no detectable effect on adaptive immune response in our experimental conditions.

Cochlin Cleavage Products Are Released in the Blood during Inflammation

Because the conduit lumens connect with the blood circulation (Gretz et al., 2000), we next tested whether cochlin is released into the bloodstream by using immunoblot analysis of the serum for detection. In basal conditions, two weak immunoreactive bands reacting with the cochlin antibody were present in the blood, which were absent in the sera from *Coch*^{-/-} mice (Figure 3A). We named them p8 and p18 according to their respective apparent molecular weights. Because the *Limulus* Factor C, which contains a homologous LCCL domain, is secreted into the

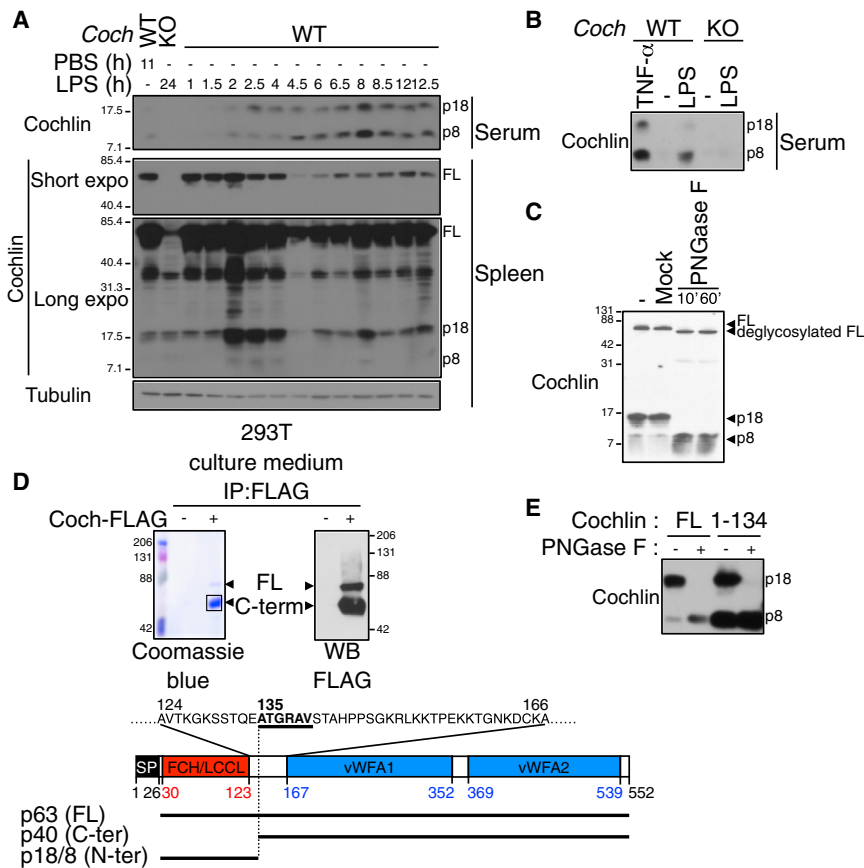


Figure 3. Cochlin LCCL Domain Is Released into the Blood following Proteolytic Cleavage upon Inflammation

(A and B) WT and *Coch*^{-/-} mice were injected i.p. with LPS (5 mg/kg) (A), TNF- α (15 μ g, 8 hr) (B), or PBS as control.

(C) The p8 fragment is the deglycosylation product of p18. Serum-free culture medium from 293T cells expressing cochlin was treated by PNGase F for 1 hr (-, untreated; mock, 1 hr without PNGase F). (D) The C-term fragment corresponds to aa 135–552. Anti-FLAG IPs from the culture medium of 293T cells expressing cochlin-FLAG.

(E) The p8 and p18 fragments correspond to aa 26–134. Serum-free culture media of 293T cells expressing the full-length or the N-terminal domain (aa 1–134) of cochlin treated with PNGase F as indicated.

See also Figure S3.

hemolymph in response to LPS (Iwanaga et al., 1992), we next tested whether cochlin p8 and p18 are released into the blood during LPS response. We found that the levels of cochlin p8 and p18 in sera greatly increased shortly after intraperitoneal (i.p.) injection of LPS in WT but not *Coch*^{-/-} mice (Figure 3A). In accordance with cleavage followed by secretion, full-length cochlin is substantially reduced in the spleen starting 4 hr after LPS injection, and p18 peaks transiently in the spleen from 2 hr to 4 hr following LPS injection before gradually decreasing but being maintained at a detectable level. Altogether these data suggest that full-length cochlin is processed into the p8 and p18 forms in the spleen prior to their release in the blood.

The levels of tumor necrosis factor- α (TNF- α), one of the first cytokines detectable in the blood, peaked at 1 hr after LPS injection (Figure S4A). We observed that TNF- α injection is sufficient to induce the release of the cochlin p8 and p18 forms into the blood (Figure 3B). This suggests that cochlin p18 and p8 release in the blood is mediated downstream of TNF- α release following LPS injection. Thus, increased levels of cochlin p8 and p18 in the blood are not restricted to LPS stimulation per se but may be a common phenomenon under various inflammatory conditions involving TNF- α .

The Cochlin p8 and p18 Forms Correspond to the LCCL Domain

Because the cochlin antibody used for the WB analysis recognizes the N-terminal part of cochlin (Yao et al., 2010), which con-

tains a LCCL domain, the p8 and p18 are most likely derived from the LCCL domain. Consistent with this possibility, we have previously shown that cochlin p8 and p18 forms are detectable in conditioned media of 293T cells transfected with a cochlin expression vector (Yao et al., 2010). To further characterize the identity of the cochlin p8 and p18, we treated conditioned media of 293T cells expressing cochlin with N-glycosidase F, because cochlin is known to be glycosylated (Kommareddi et al., 2007; Robertson et al., 2003). We observed that following N-glycosidase F treatment, the cochlin p18 disappeared while the p8 level correspondingly increased (Figure 3C), suggesting that cochlin p18 is the glycosylated form of p8.

We then determined the cleavage site of full-length cochlin by Edman degradation of the purified C-terminal FLAG-tagged cleavage product corresponding to that of p18 (Figure 3D); “AT-GRV” was found to be its N-terminal residues. Because this sequence corresponds to aa 135–140 of full-length cochlin, we concluded that cochlin is cleaved between E134 and A135. Cochlin p8 and p18 fragments are recognized by the monoclonal antibody 9A10D2 raised against the N-terminal cochlin antigen corresponding to aa 7–227 (Yao et al., 2010). Because aa 1–25 correspond to the signal peptide, we hypothesized that the p8 and p18 N-terminal fragment may correspond to the aa 26–134. We compared the migration profile in a SDS-PAGE gel of p8 and p18 in conditioned media of 293T cells expressing full-length cochlin and in conditioned media of 293T cells expressing cochlin aa 1–134 following PNGase F treatment. The expression from the vector coding for cochlin aa 1–134 resulted in bands of the same apparent molecular weights as p8 and p18. Taken together, we concluded that p8 corresponds to cochlin N-terminal fragment aa 26–134, which is then glycosylated to form p18 (Figure 3E).

We next investigated the release of the C-terminal domain by using the polyclonal antibody recognizing epitopes

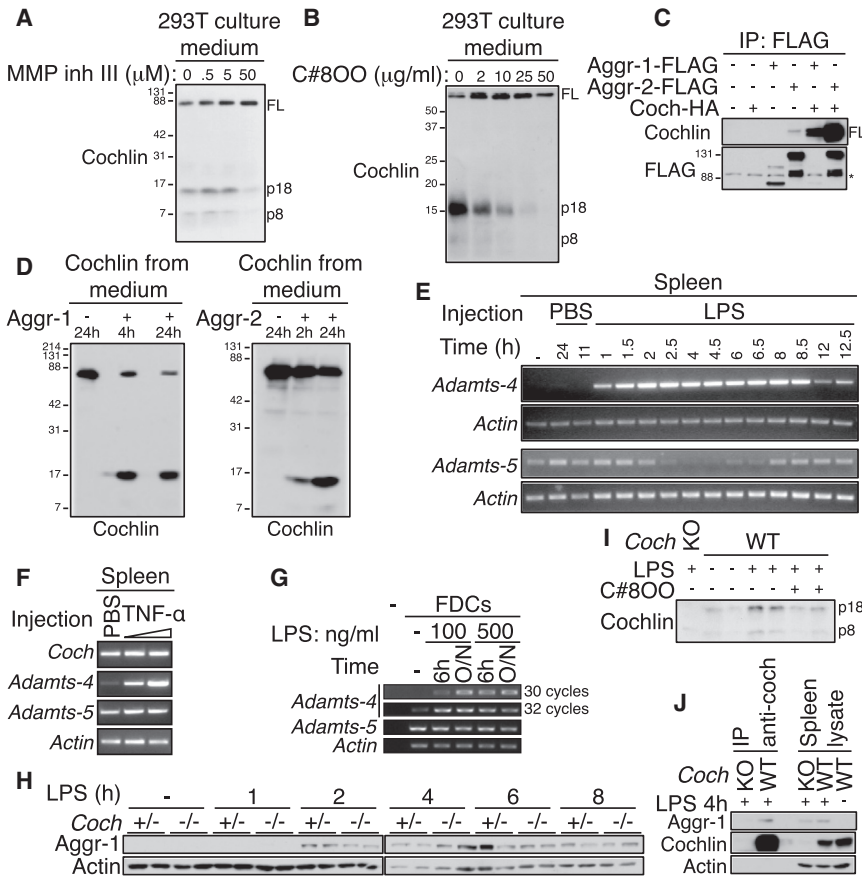


Figure 4. Cochlin Is Cleaved by the Metalloproteases Aggrecanase-1 and Aggrecanase-2

(A and B) Aggrecanase inhibitors block cochlin cleavage. 293T cells expressing cochlin were cultured in serum-free medium with the indicated concentrations of MMP inhibitor III or compound 800 for 24 hr.

(C) Cochlin interacts with aggrecanase-1 and aggrecanase-2. Anti-FLAG IP from lysates of 293T cells expressing the indicated constructs (*, nonspecific band) is shown.

(D) Aggrecanase-1 and aggrecanase-2 cleave secreted full-length cochlin. Purified full-length cochlin was treated with aggrecanase-1 (left panel) or aggrecanase-2 (right panel).

(E and F) *Adamts-4* gene expression is upregulated in mice spleen upon LPS or TNF- α injection. Spleens from mice injected i.p. with LPS (5 mg/kg) (E), mTNF- α (5 μ g or 15 μ g, 8 hr), (F) or PBS (as control) were analyzed by RT-PCR for the expression of *Adamts-4* and *Adamts-5*.

(G) Aggrecanase-1 and aggrecanase-2 are expressed by FDCs. In vitro FDC cultures were treated with LPS (100 ng/ml, 6 hr or overnight [O/N]). Aggrecanase-1 and aggrecanase-2 expressions were analyzed by RT-PCR.

(H) Aggrecanase-1 protein level is upregulated in the spleen upon LPS injection with kinetics similar to cochlin cleavage. Spleens from *Coch*^{+/-} and *Coch*^{-/-} mice injected i.p. with LPS (5 mg/kg) were analyzed by WB.

(I) Aggrecanase-1- and aggrecanase-2-specific inhibitor C800 inhibits LPS-induced p18 release into the blood. WT and *Coch*^{-/-} mice were injected i.p. with LPS

(1 mg/kg) and C800 (100 mg/kg) or vehicle only (1.3% DMSO+1.3% solutol) for 8 hr. n = 2 mice per group.

(J) Aggrecanase-1 interacts with cochlin in vivo upon LPS injection. Spleen lysates from WT and *Coch*^{-/-} mice injected i.p. with LPS (5 mg/kg, 4 hr) were subjected to anti-cochlin IP.

See also Figure S4.

predominantly in this domain. The C-terminal domain remained undetectable in the serum even after LPS injection (Figure S4B), and IF staining using the same antibody showed no decrease in cochlin intensity in the spleen following LPS injection (Figures S4C and S4D). We concluded that, contrary to the LCCL domain, the C-terminal domain of cochlin remains in the spleen following cochlin cleavage.

To determine the expression of LCCL domains in sera under control or LPS stimulated conditions, we next used purified cochlin aa 26–134 fused with hemagglutinin (HA) (hereafter named LCCL-HA) of known concentration as a standard. We found that the expression of p8 and p18 under basal conditions are too low to be precisely determined, whereas 8 hr after LPS injection the concentration of p8 and p18 reach ~0.4 μ g/ml (Figures S4E and S4F).

Together these data demonstrate that cochlin LCCL domain is released from the spleen into the blood by cochlin proteolytic cleavage during inflammation.

Cochlin Is Processed by Aggrecanase-1 and Aggrecanase-2

To explore the mechanism that mediates the cleavage of full-length cochlin, we incubated 293T cells expressing cochlin

with chemical inhibitors of different proteases. We found that in the presence of MMP inhibitor III, a broad spectrum metalloprotease inhibitor, no p8 and p18 fragments were detected in the culture medium of 293T cells expressing cochlin (Figure 4A). Because cochlin is cleaved between E134 and A135, we next investigated whether two glutamyl endopeptidases in the metalloprotease family known to cleave E-A bonds, namely aggrecanase-1 and aggrecanase-2, were involved in processing cochlin (Nagase and Kashiwagi, 2003). Compound 800, a specific inhibitor of aggrecanases (patent US2007/0043066) inhibits the release of p8 and p18 fragments in the culture medium of 293T cells expressing cochlin (Figure 4B). In addition, we observed that aggrecanase-1 and aggrecanase-2 physically interact with cochlin as shown by coimmunoprecipitation assay in 293T cells (Figure 4C) and that purified aggrecanase-1 and aggrecanase-2 can directly cleave full-length cochlin to the cochlin p18 fragment in an in vitro cleavage assay (Figure 4D).

To investigate the mechanism that leads to the processing and secretion of cochlin following LPS or TNF- α stimulation, we assessed aggrecanase expression in the spleen following LPS or TNF- α injection. The *Adamts-4* (a desintegrin and metalloproteinase with thrombospondin motifs) gene encoding aggrecanase-1 is not expressed in the spleen in basal conditions

but its transcription is greatly activated as soon as 1 hr after LPS injection (Figure 4E). *Adamts-4* mRNA was also induced following TNF- α injection (Figure 4F). On the other hand, *Adamts-5* gene encoding aggrecanase-2 is expressed in the spleen under basal conditions. However, the expression of *Adamts-5* not only is not activated following LPS or TNF- α injection but actually decreases transiently following LPS injection (Figures 4E and 4F). Consistently, the expression of *Adamts-4* mRNA in cultured FDCs was induced by LPS, whereas that of *Adamts-5* mRNA in FDCs was constitutive (Figure 4G).

At the protein level, splenic aggrecanase-1 was induced 2 hr after LPS injection, corresponding exactly to the kinetics of the observed cochlin cleavage in the spleen and the secretion of cochlin p8 and p18 into the blood (Figures 4H and 3A). Consistent with the processing of cochlin by aggrecanases, the levels of p8 and p18 induced in the serum by LPS were reduced by the injection of compound 800 (Figure 4I). We next tested the interaction of endogenous cochlin with aggrecanase-1 in vivo. Aggrecanase-1 coimmunoprecipitates with cochlin in spleen lysates from WT mice injected with LPS (Figure 4J). Neither aggrecanase-1 nor cochlin bands were detectable in the anticochlin immunoprecipitation from the splenic lysate of a *Coch*^{-/-} mouse treated in the same conditions, demonstrating the specificity of the assay. Together with the in vitro data, our results strongly indicate that aggrecanase-1 cleaves cochlin in vivo during LPS and TNF- α responses.

Cochlin Deficiency Leads to Defects in Antibacterial Innate Immunity

To characterize the role of cochlin in antibacterial innate immunity, we first examined the role of cochlin in LPS responses, based on the homology of its LCCL domain with the evolutionarily ancient related Factor C protein from the horseshoe crab *Limulus*. Factor C is critical in sensing LPS and in the consequent activation of the hemolymph coagulation cascade, as well as in complement deposition onto the surface of bacteria (Ariki et al., 2008; Iwanaga et al., 1992; Koshiba et al., 2007). However, we did not detect any interaction between the cochlin LCCL domain and LPS (data not shown). *Coch*^{-/-} macrophages showed no defect in the response to LPS in vitro, nor did the addition of recombinant cochlin LCCL domain have any impact on this response (Figures S5A and S5B). Moreover, *Coch*^{-/-} mice showed normal coagulation and cytokine responses to LPS injection in vivo (Figures S5C and S5D), and their viability to LPS challenge was not modified as compared to that of WT mice (Figure S5E). We thus concluded that cochlin is not involved in the control of the LPS response.

We next tested whether cochlin took part in the detection of bacteria by pattern recognition receptors (PRRs) independently of LPS recognition by TLR4-MD2. In vitro, *Coch*^{-/-} macrophages respond normally to peptidoglycans (PGN), which activate TLR2 and Nod-1 and Nod-2 receptors, and the addition of recombinant cochlin LCCL domain did not impact this response (Figure S5A). We next investigated the role of cochlin in the detection of intact bacteria by using *P. aeruginosa* (strain PA14) as a model. Because *Coch*^{-/-} macrophages, in the presence or absence of recombinant cochlin LCCL domain, showed no difference in cytokine responses to the infection by PA14

in vitro (Figure S5F), we concluded that cochlin is not involved in the mechanism of bacterial detection per se.

We next reasoned that while the molecular mechanisms used by cochlin and *Limulus* Factor C might have diverged, their function might nevertheless be conserved, and we investigated the role of cochlin in response to bacteria in vivo. We examined how cochlin might impact infection caused by *P. aeruginosa*, a gram-negative bacteria usually not pathogenic in healthy individuals but which constitutes a major cause of morbidity for cystic fibrosis patients with chronic lower respiratory tract infection, as well as acute opportunistic pneumonias in immunocompromised individuals (Sadikot et al., 2005). Innate immune responses that recruit neutrophils to the lungs are the primary means by which mammals resist *P. aeruginosa* infections (Koh et al., 2009). Similar to that of LPS injection, intranasal (i.n.) infection with *P. aeruginosa* (strain PA14) triggered the release of cochlin p8 and p18 into mouse sera (Figure 5A). More importantly, 24 hr after i.n. infection of PA14, *Coch*^{-/-} mice showed higher bacterial loads in the lungs, blood, and spleens compared to WT mice (Figure 5B). This defect in controlling infection with *P. aeruginosa* was associated with higher levels of cytokines such as IL-6 and IL-18 in the serum at 24 hr (Figure 5C) and decreased survival of *Coch*^{-/-} mice (Figure 5D). Similarly, *Coch*^{-/-} mice showed higher bacterial loads in the lungs, blood, and spleens as compared to WT mice 24 hr following i.n. infection with the gram-positive bacteria *Staphylococcus aureus* (LAC strain) (Figure 5E), demonstrating that cochlin antibacterial function is not restricted to one specific bacteria strain, and definitively excluding a LPS-dependent mechanism. Because cochlin LCCL had direct effect neither on the in vitro viability and growth of PA14 and LAC nor on biofilm formation (Figures S5G and S5J), we concluded that cochlin is unlikely to be a bactericidal effector and hypothesized that it may regulate innate immune response. Indeed, analysis of the bronchoalveolar lavage of infected lungs showed that *Coch*^{-/-} mice had a defect in the local secretion of IL-6, TNF- α , IL-1 β , and in the macrophage and neutrophil chemoattractants MCP-1 and KC at 8 hr postinfection with PA14 (Figure 5F), followed by a defective recruitment of monocytes/small macrophages and neutrophils at 12 hr after infection (Figure 5G). Differentiated alveolar macrophage counts were similar in BAL of WT and *Coch*^{-/-} mice at this time point (data not shown). Because cochlin was not expressed at the mRNA level in lung under basal condition nor following infection with PA14 (Figure 5H) but cochlin LCCL protein accumulated in the lung following infection with PA14 (Figure 5I), we hypothesized that cochlin cleavage by aggrecanases and the subsequent release of cochlin LCCL into the blood was necessary for cochlin to control bacteria loads in the lung. Indeed inhibition of aggrecanases by i.v. injection of compound 800 greatly increased lung bacteria loads, demonstrating that aggrecanase activity participate in the control of bacteria growth (Figure 5J). More importantly, the effect of compound 800 was significantly attenuated in *Coch*^{-/-} mice compared to that of WT mice as shown by the statistical significance of the interaction in the two-way ANOVA. We thus conclude that cochlin cleavage accounts for a significant part of the antibacterial effect by aggrecanases, although aggrecanases also control bacteria growth through additional cochlin-independent mechanisms.

Taken together, we conclude that cochlin, specifically expressed by the follicular dendritic cells and selectively localized

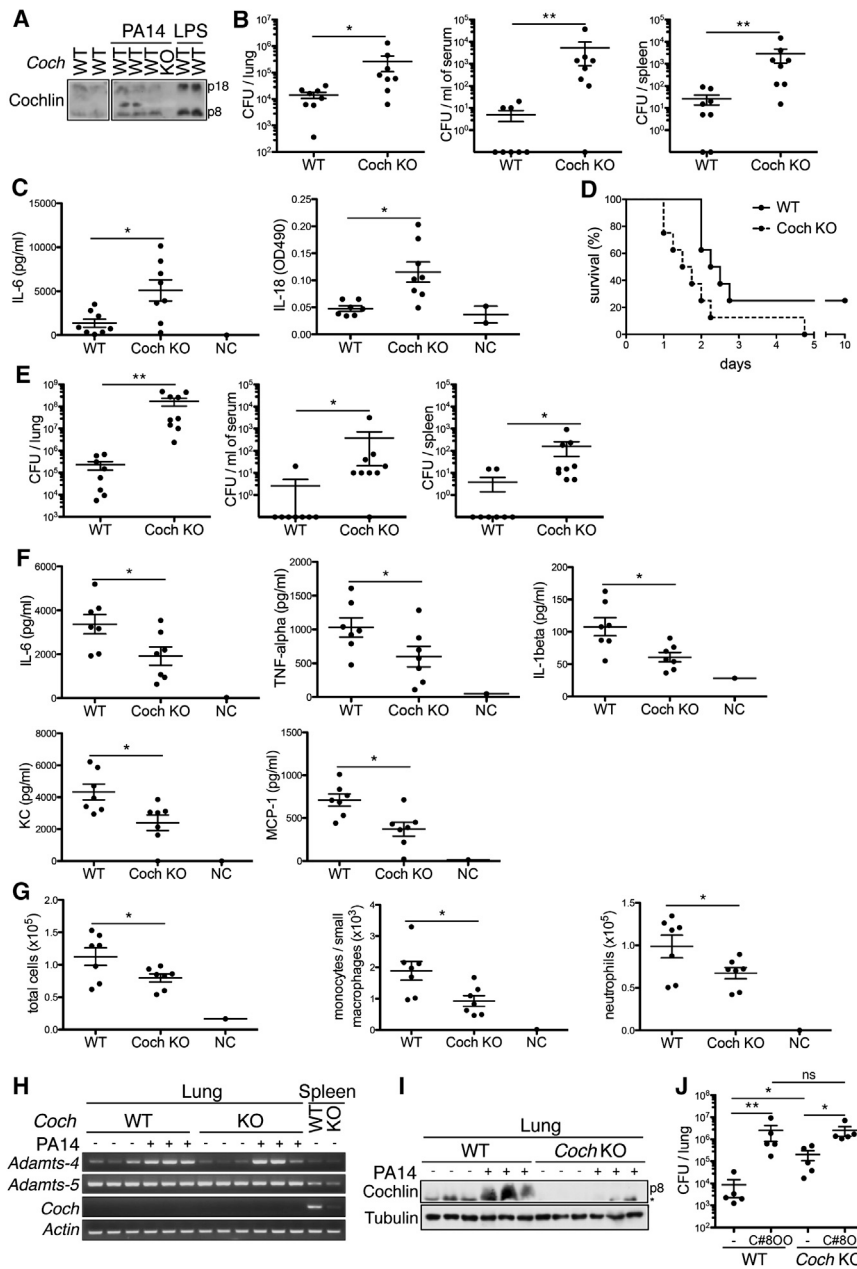


Figure 5. Cochlin Promotes Antibacterial Immunity against *P. aeruginosa*

(A) Release of cochlin p8 and p18 into the serum during infection with PA14. Sera from WT and *Coch*^{-/-} mice i.n. infected with PA14 for 10 hr were analyzed by WB. Sera from mice injected i.p. with LPS (5 mg/kg, 8 hr) were used as positive controls.

(B) Increased CFU number in lung and bacterial spread in *Coch*^{-/-} mice. *Coch*^{-/-} and WT mice were i.n. infected with PA14 for 24 hr. n = 8 per group; *p < 0.01; **p < 0.001; data are representative of four independent experiments. Error bars represent 1 SEM.

(C) Enhanced systemic cytokine burst in *Coch*^{-/-} mice. *Coch*^{-/-} and WT mice were i.n. infected with PA14 for 24 hr. IL-6 and IL-18 concentrations in sera were measured by ELISA. Uninfected mice were used as negative controls (NC). n = 8 per group; *p < 0.01. Error bars represent 1 SEM.

(D) Reduced survival of infected *Coch*^{-/-} mice. *Coch*^{-/-} and WT mice were i.n. infected with PA14. n = 8 per group; Gehan-Breslow-Wilcoxon test p < 0.05; data are representative of two independent experiments.

(E) Increased CFU number in lung and bacterial spread in *Coch*^{-/-} mice following *S. aureus* i.n. infection. *Coch*^{-/-} and WT mice were i.n. infected with LAC for 24 hr. n = 8–9 per group; *p < 0.01; **p < 0.001; data are representative of two independent experiments. Error bars represent 1 SEM.

(F) Decreased cytokine levels in the BAL fluid of *Coch*^{-/-} mice. *Coch*^{-/-} and WT mice were i.n. infected with PA14 for 8 hr. Cytokines concentrations in the BAL fluid were determined by ELISA. n = 7 per group; *p < 0.05. Error bars represent 1 SEM.

(G) Decreased lung recruitment of PMNs in *Coch*^{-/-} mice. *Coch*^{-/-} and WT mice were i.n. infected with PA14 for 12 hr. Cell numbers and composition in the BAL was analyzed by flow cytometry. Neutrophils are defined as CD11c^{neg}, CD11b^{high} and GR-1^{pos}. Monocytes and small macrophages are defined as CD11c^{neg}, CD11b^{mild}, and GR-1^{neg}. n = 7 per group; *p < 0.05. Error bars represent 1 SEM.

(H and I) Cochlin is not produced but LCCL accumulates in the lung following *P. aeruginosa* i.n. infection. *Coch*^{-/-} and WT mice were infected with PA14 for 8 hr. Lung were analyzed for cochlin mRNA expression by RT-PCR (H) and cochlin LCCL protein accumulation by WB (I). *, unspecific band.

(J) Reduced effect of aggrecanase inhibition on CFU number in the lung of *Coch*^{-/-} mice. *Coch*^{-/-} and WT mice were i.v. injected with C800 (100 mg/kg) or vehicle only (–) and i.n. infected with PA14 for 24 hr. n = 5 per group; *p < 0.01; **p < 0.001; ns, nonsignificant. The effect of cochlin deficiency on the increase in CFU following C800 injection was assessed by a two-way ANOVA by using “genotype,” “C800 treatment,” and the interaction between “genotype” and “C800 treatment” as fixed factors. “genotype”: F_{1,18} = 11.68, p = 0.0035; “C800”: F_{1,18} = 55.07, p < 0.001; “genotype*C800”: F_{1,18} = 5.57, p = 0.0313. Error bars represent 1 SEM.

See also Figure S5.

in the fine extracellular network of conduits in the spleen and lymph nodes, is an important modulator of innate immune response. During infection and inflammation, cochlin is cleaved by aggrecanases and secreted into blood circulation and regulates local cytokine production, recruitment of immune effector cells, and bacterial clearance (Figure S5K). We propose that by regulating the production of cochlin, the FDCs may have a previously unknown function in innate immune response in defense against bacteria.

DISCUSSION

Our study describes cochlin as a modulator of innate immunity produced by the FDCs, the known critical modulators of humoral immunity promoting activation and maturation of B cells within follicles (Allen and Cyster, 2008). FDCs are tightly associated with conduits, a fine extracellular network in spleen and lymph nodes that is involved in small antigens and cytokines transport. While other conduit-associated cytokines have been shown to

regulate local cell migration within the lymphoid organ, our study demonstrates cochlin as the first immune modulator locally deposited by the FDCs into the conduits and released upon inflammation into the systemic circulation. Our study supports the notion that the conduit is not only a channel mediating passive diffusion of small molecules, but also a reservoir for ready-to-be-released immune regulators to play active roles in modulating immunity. Moreover, our study suggests a unique role for the FDCs in modulating innate immunity.

We identified the FDCs as the producer of cochlin in the secondary lymphoid organs. FDCs play a critical role in germinal center formation and B cell activation, affinity maturation, and differentiation (Allen and Cyster, 2008). *Coch*^{-/-} mice showed no defects in germinal center formation, immunoglobulin responses, or antibody affinity maturation, suggesting that cochlin in the FDCs may serve no function with regards to B cell activation. A previous study implicated cochlin in cell-adhesion, most likely through its two C terminus von Willebrand factor A-like (vWFA) domains (Bhattacharya et al., 2005). However, cochlin deficiency does not modify FDC localization around the conduits or their ability to capture antigen flowing in the conduit's lumen, suggesting that cochlin does not play a critical role in mediating adhesion of FDCs. Although cochlin is associated with structures mediating fluid flow such as the trabecular meshwork in the eyes, the conduits in the secondary lymphoid organs, as well as in the inner ear (Bhattacharya et al., 2005; Nolte et al., 2003; Robertson et al., 2006; Roozendaal et al., 2009), cochlin-deficiency causes no impairment in the flow inside the conduit, suggesting that cochlin does not play a critical structural role in supporting the integrity of the fine tubular structure. Because abnormal accumulation of cochlin in the inner ear and the eyes has been proposed to interfere with fluid flow and contribute to pathological conditions (Bhattacharya et al., 2005), a possible future study may be to identify any condition where abnormal accumulation of cochlin in the conduits may interfere with the flow, which might provide further insights for the physiological function of the conduits in the secondary lymphoid organs.

Our fluorescent and electron microscopy data consistently show that cochlin colocalizes with collagen-forming fibrillar and bundle structures in the conduits. A similar association with type II fibrillar collagen has previously been shown in the inner ear (Mizuta et al., 2008). Indeed, type I, II, and IV collagens bind to the second vWFA domain but not the LCCL-domain of cochlin (Nagy et al., 2008). Our data reveal that the LCCL domain is released in the blood following cochlin cleavage between its LCCL domain and its C-terminal domain containing the two vWFA domains. While cochlin C-terminal domains (named p40 and p45) are found in the spleen (Rodriguez et al., 2004), they remained undetectable in the blood in both basal and inflammatory conditions. Thus, our working model is that cochlin binds to conduit collagen through its vWFA domains, and following cochlin cleavage by aggrecanases, the LCCL domain is released into the bloodstream while the C-terminal domain remained bound to collagen in the conduits.

Multiple cochlin isoforms have been described that result from both alternative splicing and posttranslational modifications, and their expression patterns show tissue specificity (Bhattacharya et al., 2005; Ikezono et al., 2001; Kommareddi et al., 2007; Mizuta et al., 2008; Robertson et al., 2001). Our study reports p8 and

p18 as new circulating forms of cochlin, highly reminiscent of the N-terminal p16 fragment named cochlin tomoprotein (CTP) recently identified in the ear perilymph, but absent from inner ear tissue (Ikezono et al., 2004). Our data open perspectives in the identification of CTP, which may be identical to p8 and p18, and in its mechanism of secretion that may be dependent on aggrecanases. In addition, our finding that p18 is a glycosylated product of p8 is consistent with the residue N102 being a consensus site for N-linked glycosylation.

Our observation that the cochlin cleavage site [E-AtgRavSTA] was highly similar to the established aggrecanase-1 consensus cleavage site [E-(AFVLMY)-X(0,1)-(RK)-X(2,3)-(ST)-(VYIFWMLA)] led us to identify cochlin as a new substrate for aggrecanase-1 and aggrecanase-2. These glutamyl-endopeptidases from the ADAMTS metalloprotease family play a key role in arthritis pathogenesis by cleaving the cartilage matrix proteoglycan aggrecan (Lin and Liu, 2010). The other substrates of the ADAMTS family include several components of the extracellular matrix such as COMP (cartilage oligomer protein), biglycan, TIMP-4, matrilin-2, and matrilin-3 (Hills et al., 2007). We found that aggrecanase-2, but not aggrecanase-1, is constitutively expressed in the spleen, suggesting that the low basal circulating level of cochlin p8 and p18 in the blood may result of aggrecanase-2 activity. Aggrecanase-1 expression is highly induced in the spleen following LPS or TNF- α injection with kinetics matching exactly these of cochlin cleavage. This observation is consistent with multiple reports claiming that aggrecanase-1 expression is upregulated by inflammatory cytokines (Bondeson et al., 2006; Cross et al., 2006; Song et al., 2007; Tortorella et al., 2001; Yamanishi et al., 2002).

Our discovery that cochlin LCCL domain is a blood-circulating protein with increased level during inflammation led us to investigate a systemic role of the LCCL domain in the innate immune response. LCCL is an autonomous folding domain consisting of a central α helix wrapped by two β sheets named after the first three proteins identified to contain this domain (Limulus factor C, Cochlin and Lgl-1 [late gestation lung]) and later found in various secreted proteins with modular structures (Liepinsh et al., 2001). The horseshoe crab *Limulus* coagulation factor C is a serine protease secreted in the hemolymph, activated upon LPS binding, and is critical in antibacterial immunity. In particular, factor C mediates the degranulation of the amoebocytes, the initiation of a protease cascade resulting in coagulation, and the activation of the complement system (Ariki et al., 2008; Iwanaga et al., 1992; Koshiba et al., 2007). The contribution of the LCCL domain in the function of factor C remains unknown, and in particular LCCL does not bind LPS (Koshiba et al., 2007; Tan et al., 2000). A role in antibacterial innate immunity has also been suggested for the mammalian protein Igl-1 (also named CRISPLD2 for cysteine-rich secretory protein LCCL domain containing 2) expressed in various organs and notably detected in the serum. Lgl-1 binds to LPS and sequesters it away from the PBMCs surface (Wang et al., 2009). Cochlin LCCL sequence and secretion pattern homologies with *Limulus* factor C and Igl-1 led us to hypothesize a conservation of their functions. Indeed, we discovered that cochlin is critical in innate immunity by using models of *P. aeruginosa* and *S. aureus* i.n. infection. However, contrary to factor C or Igl-1, cochlin effector function is independent of LPS because cochlin LCCL domain does not bind to LPS (data not shown; Liepinsh et al., 2001), and impacts LPS response neither in vitro nor in vivo. In addition, the low affinity of

the *P. aeruginosa* LPS for TLR4 and the absence of phenotype of the *Tlr4*^{-/-} mice in regards to *P. aeruginosa* infection predict that the TLR4-mediated LPS response is accessory in the immunity against this pathogen, and modulation of this pathway is not expected to impact the outcome of the infection (Feuillet et al., 2006; Ramphal et al., 2005). Finally cochlin role in controlling the gram-positive bacteria *S. aureus* definitely demonstrates a LPS-independent mechanism.

Our study shows a defect of *Coch*^{-/-} mice in local cytokine and chemoattractant production in the lung and in the subsequent recruitment of immune cells leading to an impaired control of the bacteria and a consequent reduced viability following infection. These data confirm previous studies describing that the recruitment of neutrophils and macrophages to the lung is absolutely critical for the outcome of *P. aeruginosa* infection and that blunted early local inflammatory response in the lung leads to later higher bacteria load, uncontrolled systemic cytokine secretion and reduced survival (Horino et al., 2009; Koh et al., 2009). Taken together, our study demonstrates an unexpected function of FDC in modulating innate immunity and a role for secondary lymphoid organ and particularly local lymph nodes in the amplification of the innate immune response by the release of modulators such as cochlin.

EXPERIMENTAL PROCEDURES

Expression Vectors

Cochlin expression vectors have already been described (Yao et al., 2010). *Adamts-4* and *Adamts-5* genes were cloned from total cDNA isolated from C57B6 mouse spleen by using the primers *adamts4-f* 5'-CATTTTGGTGCCG CAGATG-3', *adamts4-r* 5'-CGGGACAGTGAGGTTATTTC-3', *adamts5-f* 5'-CACTATGCGGCTCGAGTG-3', *adamts5-r* 5'-CAGGCTAACATTTCTTCAG CAGAC-3' in pcDNA3.

Reagents

Compound 800 (patent US2007/0043066) was custom synthesized by Shanghai ChemPartner.

Antibodies

The monoclonal antiochlin 9A10D2 raised against aa 7–227 of cochlin has been described (Yao et al., 2010) (WB analysis). Rabbit polyclonal antibody P13 was raised against cochlin aa 7–227 (histology staining and immunoprecipitation). The rat monoclonal AD4/4D2E10 antiaggrecanase-1 was raised against full-length murine aggrecanase-1.

FDC Isolation

FDCs were isolated from LN as described (Sukumar et al., 2006) and cultured in FDCs media (DMEM, FCS 10%, HEPES 20 mM, glutamine 2 mM, gentamicin 50 µg/ml, nonessential amino acid 1X, β-mercaptoethanol 4 × 10⁻⁴%).

In Vitro Cleavage Assay

The C-term FLAG-tagged aggrecanase-1 and aggrecanase-2 were purified by IP from culture media of 293T cells transfected with the murine *Adamts-4* (aggr-1) or *Adamts-5* (aggr-2) constructs. The full-length C-term FLAG-tagged cochlin was similarly purified by IP from culture media of transfected 293T cells in the presence of MMP inhibitor III (50 µM). Proteins were eluted by FLAG peptide and dialyzed in cleavage buffer (Tris HCl pH 7.5 50 mM, NaCl 100 mM, CaCl₂ 10 mM, Na₃N 0.02%, NP-40 0.02%). Cochlin and aggrecanase-1 or aggrecanase-2 were incubated in cleavage buffer at 37°C, and reactions were stopped by Laemmli buffer.

Bacteria Infection

Gender-matched 6- to 9-week-old *Coch*^{-/-} and C57B6 control mice were sedated with ketamine hydrochloride (65 mg/kg) and xylazine (13 mg/kg)

and infected i.n. with PA14 (1–3 × 10⁷ CFU) or LAC (1–3 × 10⁸ CFU). For CFU numeration, lungs, blood, and spleens were harvested and homogenized in LB, and dilutions were plated on LB-agar plates. Bronchoalveolar lavages (BAL) were collected in 2 ml of PBS and spun at 1,500 rpm for 5 min.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2013.01.015>.

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REFERENCES

- Allen, C.D., and Cyster, J.G. (2008). Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function. *Semin. Immunol.* *20*, 14–25.
- Ariki, S., Takahara, S., Shibata, T., Fukuoka, T., Ozaki, A., Endo, Y., Fujita, T., Koshiba, T., and Kawabata, S. (2008). Factor C acts as a lipopolysaccharide-responsive C3 convertase in horseshoe crab complement activation. *J. Immunol.* *181*, 7994–8001.
- Bajénoff, M., Egen, J.G., Koo, L.Y., Laugier, J.P., Brau, F., Glaichenhaus, N., and Germain, R.N. (2006). Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. *Immunity* *25*, 989–1001.
- Bhattacharya, S.K., Rockwood, E.J., Smith, S.D., Bonilha, V.L., Crabb, J.S., Kuchtey, R.W., Robertson, N.G., Peachey, N.S., Morton, C.C., and Crabb, J.W. (2005). Proteomics reveal Cochlin deposits associated with glaucomatous trabecular meshwork. *J. Biol. Chem.* *280*, 6080–6084.
- Bondeson, J., Wainwright, S.D., Lauder, S., Amos, N., and Hughes, C.E. (2006). The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. *Arthritis Res. Ther.* *8*, R187.
- Cross, A.K., Haddock, G., Stock, C.J., Allan, S., Surr, J., Bunning, R.A., Buttle, D.J., and Woodroffe, M.N. (2006). ADAMTS-1 and -4 are up-regulated following transient middle cerebral artery occlusion in the rat and their expression is modulated by TNF in cultured astrocytes. *Brain Res.* *1088*, 19–30.
- El Shikh, M.E., El Sayed, R.M., Wu, Y., Szakal, A.K., and Tew, J.G. (2007). TLR4 on follicular dendritic cells: an activation pathway that promotes accessory activity. *J. Immunol.* *179*, 4444–4450.
- Feuillet, V., Medjane, S., Mondor, I., Demaria, O., Pagni, P.P., Galán, J.E., Flavell, R.A., and Alexopoulou, L. (2006). Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria. *Proc. Natl. Acad. Sci. USA* *103*, 12487–12492.
- Garin, A., Meyer-Hermann, M., Contie, M., Figge, M.T., Buatois, V., Gunzer, M., Toellner, K.M., Elson, G., and Kosco-Vilbois, M.H. (2010). Toll-like receptor 4 signaling by follicular dendritic cells is pivotal for germinal center onset and affinity maturation. *Immunity* *33*, 84–95.
- Gretz, J.E., Norbury, C.C., Anderson, A.O., Proudfoot, A.E., and Shaw, S. (2000). Lymph-borne chemokines and other low molecular weight molecules reach high endothelial venules via specialized conduits while a functional

- barrier limits access to the lymphocyte microenvironments in lymph node cortex. *J. Exp. Med.* 192, 1425–1440.
- Hills, R., Mazzarella, R., Fok, K., Liu, M., Nemirovskiy, O., Leone, J., Zack, M.D., Arner, E.C., Viswanathan, M., Abujoub, A., et al. (2007). Identification of an ADAMTS-4 cleavage motif using phage display leads to the development of fluorogenic peptide substrates and reveals matrilin-3 as a novel substrate. *J. Biol. Chem.* 282, 11101–11109.
- Horino, T., Matsumoto, T., Ishikawa, H., Kimura, S., Uramatsu, M., Tanabe, M., Tateda, K., Miyazaki, S., Aramaki, Y., Iwakura, Y., et al. (2009). Interleukin-1 deficiency in combination with macrophage depletion increases susceptibility to *Pseudomonas aeruginosa* bacteremia. *Microbiol. Immunol.* 53, 502–511.
- Ikezono, T., Omori, A., Ichinose, S., Pawankar, R., Watanabe, A., and Yagi, T. (2001). Identification of the protein product of the Coch gene (hereditary deafness gene) as the major component of bovine inner ear protein. *Biochim. Biophys. Acta* 1535, 258–265.
- Ikezono, T., Shindo, S., Li, L., Omori, A., Ichinose, S., Watanabe, A., Kobayashi, T., Pawankar, R., and Yagi, T. (2004). Identification of a novel Cochlin isoform in the perilymph: insights to Cochlin function and the pathogenesis of DFNA9. *Biochem. Biophys. Res. Commun.* 314, 440–446.
- Iwanaga, S., Miyata, T., Tokunaga, F., and Muta, T. (1992). Molecular mechanism of hemolymph clotting system in *Limulus*. *Thromb. Res.* 68, 1–32.
- Koh, A.Y., Priebe, G.P., Ray, C., Van Rooijen, N., and Pier, G.B. (2009). Inescapable need for neutrophils as mediators of cellular innate immunity to acute *Pseudomonas aeruginosa* pneumonia. *Infect. Immun.* 77, 5300–5310.
- Kommareddi, P.K., Nair, T.S., Raphael, Y., Telian, S.A., Kim, A.H., Arts, H.A., El-Kashlan, H.K., and Carey, T.E. (2007). Cochlin isoforms and their interaction with CTL2 (SLC44A2) in the inner ear. *J. Assoc. Res. Otolaryngol.* 8, 435–446.
- Koshihara, T., Hashii, T., and Kawabata, S. (2007). A structural perspective on the interaction between lipopolysaccharide and factor C, a receptor involved in recognition of Gram-negative bacteria. *J. Biol. Chem.* 282, 3962–3967.
- Liepinsh, E., Trexler, M., Kaikkonen, A., Weigelt, J., Bányai, L., Patthy, L., and Otting, G. (2001). NMR structure of the LCCL domain and implications for DFNA9 deafness disorder. *EMBO J.* 20, 5347–5353.
- Lin, E.A., and Liu, C.J. (2010). The role of ADAMTSs in arthritis. *Protein Cell* 1, 33–47.
- Lokmic, Z., Lämmermann, T., Sixt, M., Cardell, S., Hallmann, R., and Sorokin, L. (2008). The extracellular matrix of the spleen as a potential organizer of immune cell compartments. *Semin. Immunol.* 20, 4–13.
- Manolis, E.N., Yandavi, N., Nadol, J.B., Jr., Eavey, R.D., McKenna, M., Rosenbaum, S., Khetarpal, U., Halpin, C., Merchant, S.N., Duyk, G.M., et al. (1996). A gene for non-syndromic autosomal dominant progressive postlingual sensorineural hearing loss maps to chromosome 14q12-13. *Hum. Mol. Genet.* 5, 1047–1050.
- Mizuta, K., Ikezono, T., Iwasaki, S., Arai, M., Hashimoto, Y., Pawankar, R., Watanabe, T., Shindo, S., and Mineta, H. (2008). Ultrastructural co-localization of cochlin and type II collagen in the rat semicircular canal. *Neurosci. Lett.* 434, 104–107.
- Nagase, H., and Kashiwagi, M. (2003). Aggrecanases and cartilage matrix degradation. *Arthritis Res. Ther.* 5, 94–103.
- Nagy, I., Trexler, M., and Patthy, L. (2008). The second von Willebrand type A domain of cochlin has high affinity for type I, type II and type IV collagens. *FEBS Lett.* 582, 4003–4007.
- Nolte, M.A., Beliën, J.A., Schadee-Eestermans, I., Jansen, W., Unger, W.W., van Rooijen, N., Kraal, G., and Mebius, R.E. (2003). A conduit system distributes chemokines and small blood-borne molecules through the splenic white pulp. *J. Exp. Med.* 198, 505–512.
- Ramphal, R., Balloy, V., Huerre, M., Si-Tahar, M., and Chignard, M. (2005). TLRs 2 and 4 are not involved in hypersusceptibility to acute *Pseudomonas aeruginosa* lung infections. *J. Immunol.* 175, 3927–3934.
- Robertson, N.G., Khetarpal, U., Gutiérrez-Espeleta, G.A., Bieber, F.R., and Morton, C.C. (1994). Isolation of novel and known genes from a human fetal cochlear cDNA library using subtractive hybridization and differential screening. *Genomics* 23, 42–50.
- Robertson, N.G., Skvorak, A.B., Yin, Y., Weremowicz, S., Johnson, K.R., Kovatch, K.A., Battey, J.F., Bieber, F.R., and Morton, C.C. (1997). Mapping and characterization of a novel cochlear gene in human and in mouse: a positional candidate gene for a deafness disorder, DFNA9. *Genomics* 46, 345–354.
- Robertson, N.G., Resendes, B.L., Lin, J.S., Lee, C., Aster, J.C., Adams, J.C., and Morton, C.C. (2001). Inner ear localization of mRNA and protein products of COCH, mutated in the sensorineural deafness and vestibular disorder, DFNA9. *Hum. Mol. Genet.* 10, 2493–2500.
- Robertson, N.G., Hamaker, S.A., Patriub, V., Aster, J.C., and Morton, C.C. (2003). Subcellular localisation, secretion, and post-translational processing of normal cochlin, and of mutants causing the sensorineural deafness and vestibular disorder, DFNA9. *J. Med. Genet.* 40, 479–486.
- Robertson, N.G., Cremers, C.W., Huygen, P.L., Ikezono, T., Krastins, B., Kremer, H., Kuo, S.F., Liberman, M.C., Merchant, S.N., Miller, C.E., et al. (2006). Cochlin immunostaining of inner ear pathologic deposits and proteomic analysis in DFNA9 deafness and vestibular dysfunction. *Hum. Mol. Genet.* 15, 1071–1085.
- Rodriguez, C.I., Cheng, J.G., Liu, L., and Stewart, C.L. (2004). Cochlin, a secreted von Willebrand factor type A domain-containing factor, is regulated by leukemia inhibitory factor in the uterus at the time of embryo implantation. *Endocrinology* 145, 1410–1418.
- Roosendaal, R., Mempel, T.R., Pitcher, L.A., Gonzalez, S.F., Verschoor, A., Mebius, R.E., von Andrian, U.H., and Carroll, M.C. (2009). Conduits mediate transport of low-molecular-weight antigen to lymph node follicles. *Immunity* 30, 264–276.
- Sadikot, R.T., Blackwell, T.S., Christman, J.W., and Prince, A.S. (2005). Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. *Am. J. Respir. Crit. Care Med.* 171, 1209–1223.
- Sixt, M., Kanazawa, N., Selg, M., Samson, T., Roos, G., Reinhardt, D.P., Pabst, R., Lutz, M.B., and Sorokin, L. (2005). The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node. *Immunity* 22, 19–29.
- Song, R.H., Tortorella, M.D., Malfait, A.M., Alston, J.T., Yang, Z., Arner, E.C., and Griggs, D.W. (2007). Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5. *Arthritis Rheum.* 56, 575–585.
- Sukumar, S., Szakal, A.K., and Tew, J.G. (2006). Isolation of functionally active murine follicular dendritic cells. *J. Immunol. Methods* 313, 81–95.
- Suzuki, K., Maruya, M., Kawamoto, S., Sitnik, K., Kitamura, H., Agace, W.W., and Fagarasan, S. (2010). The sensing of environmental stimuli by follicular dendritic cells promotes immunoglobulin A generation in the gut. *Immunity* 33, 71–83.
- Tan, N.S., Ng, M.L., Yau, Y.H., Chong, P.K., Ho, B., and Ding, J.L. (2000). Definition of endotoxin binding sites in horseshoe crab factor C recombinant sushi proteins and neutralization of endotoxin by sushi peptides. *FASEB J.* 14, 1801–1813.
- Tortorella, M.D., Malfait, A.M., Deccico, C., and Arner, E. (2001). The role of ADAM-TS4 (aggrecanase-1) and ADAM-TS5 (aggrecanase-2) in a model of cartilage degradation. *Osteoarthritis Cartilage* 9, 539–552.
- Wang, Z.Q., Xing, W.M., Fan, H.H., Wang, K.S., Zhang, H.K., Wang, Q.W., Qi, J., Yang, H.M., Yang, J., Ren, Y.N., et al. (2009). The novel lipopolysaccharide-binding protein CRISPLD2 is a critical serum protein to regulate endotoxin function. *J. Immunol.* 183, 6646–6656.
- Yamanishi, Y., Boyle, D.L., Clark, M., Maki, R.A., Tortorella, M.D., Arner, E.C., and Firestein, G.S. (2002). Expression and regulation of aggrecanase in arthritis: the role of TGF-beta. *J. Immunol.* 168, 1405–1412.
- Yao, J., Py, B.F., Zhu, H., Bao, J., and Yuan, J. (2010). Role of protein misfolding in DFNA9 hearing loss. *J. Biol. Chem.* 285, 14909–14919.